June 1, 2016

Wednesday, June 1, 2016
5:55 PM

Today, we (as an entire group) made LB agar plates using the solution from yesterday's lab session (note: I was out-of-town on May 31, 2016). The plates were used to transform DNA into the LB media plates with the antibiotic resistant gene present so the number of cells that exhibit the same gene can fluoresce and be quantified.

The media was poured into petri dishes, then solidified. The parts from the iGEM registry were cloned into DH5alpha cells (more info towards bottom). Once the parts were cloned (spent 30 minutes), the chemically competent cells were transformed into the DNA via heat shock for exactly 1 minute, then left to recover for 45 minutes.

We added beads to spread out the cells throughout the plate and they were left in the incubator.

Parts used today:

1. BBa_K1399008 (Plate 5, Well 7P) GFP (mut3b) with DAS-ssrA degradation tag - This GFP is tagged with the ssrA tag that is recognized by ClpA and ClpX. Considered 'moderately fast'

2. BBa_K1399007 (Plate 5, Well 7N) GFP (mut3b) with NYADAS-ssrA degredation tag - considered 'fast'

3. BBa_K1399006 (Plate 5, Well 7L) GFP (mut3b) with LAA-ssrA degredation tag - considered 'very fast'

4. BBa_K1399003 (Plate 5, Well 7D) RFP from Discosoma striata (coral) with LAA-ssrA degradation tag (wt) - considered 'very fast'

BBa_I732094 *- Promoter Activity Reporter (LacZ-alpha and GFP-AAV)*
Plate 3, Well 1P

BBa_I13521 - Ptet mRFP (Positive Control)

Plate 3, Well 6G

BBa_K206000 - pBAD promoter strong

Plate 3 Well 14A

BBa_K206001 - pBAD promoter weak

Plate 3, Well 14C

June 2, 2016

Thursday, June 2, 2016
1:53 PM

Want to create primers that are specific to promoter, then go through PCR to get high concentration of that promoter that can be placed into a plasmid, and once the promoter is highly expressed and transformed into the plasmid, it can be used to measure its growth phase in bacteria

Dry lab:
From the promoter sequence sheet that was compiled on Monday, we searched the NCBI database to the corresponding number, and from there we had to select the promoter region (should have been above 150 base pairs). Once we selected the hypothesized promoter region, we used the genbank data base to obtain the DNA sequence and analyzed the sequence in the registry. The e value needed to be very small to indicate less variation among the promoter sequence with the ideal Shine-Delgarno promoter sequence after comparing with all basic parts . With the very small value, we used the "BBa_" part and searched the iGEM database to see if it was a promoter or not based on the description.

Wet lab:
We added cell cultures and antibiotic into media and let them grow overnight in the incubator. Tomorrow we should expect a sample filled with bacterial cells that exhibit the antibiotic resistant gene

June 3, 2016

Friday, June 3, 2016
4:30 PM

Today, we worked on plasmid/DNA purification using the samples that we incubated overnight

Plasmid purification (general):

1. Spin/pellet, then remove the supernatant (the liquid that isn't the pellet)
2. Lyse/extract the cells
    a. Resuspend: 250 uL of Buffer 1 into 1.5 mL tube
    b. Lyse: 250 uL of Buffer 2 and invert
    c. Neutralize: 350 uL of Buffer 3 and invert
3. Spin/purify in centrifuge for 10 minutes
    a. Load supernatant onto column
        i. Bind DNA
        ii. Buffer A- inactivates enzymes
        iii. Wash buffer

iv. Elute with H2O

June 13, 2016

Tuesday, June 14, 2016
10:13 AM

Tested another agarose gel to see if enzymes work. Problem we keep getting is half of the gel is dark. We realized the problem can be solved if we run the gel for half of the time, so instead of 40 minutes run for 20 minutes.

Tomorrow:
-run another gel for only 20 minutes, use same enzymes as yesterday
-start chromosomal dna extraction
-plan to discuss application ideas for project this week

June 14, 2016

Tuesday, June 14, 2016
10:16 AM

Project application ideas:

http://physics.illinois.edu/news/story.asp?id=17784

https://cplc.illinois.edu/

We completed a chromosomal dna extraction with the bacteria culture prepared the day before. As indicated, this step extracts the chromosomal dna from E Coli that contain promoter sequences of interest so that we are able to make primers for amplification.

June 15, 2016

Thursday, June 16, 2016
1:30 PM

Group meeting:
-want to make sure GFP degradation is part of experiment

Ideally, want a promoter followed by a GFP with a tag hanging on it. The tag would say "degrade me (the entire molecule) immediately"
This is how we want the general trend of cells to go (increase in fluorescence then decrease once tag is translated)

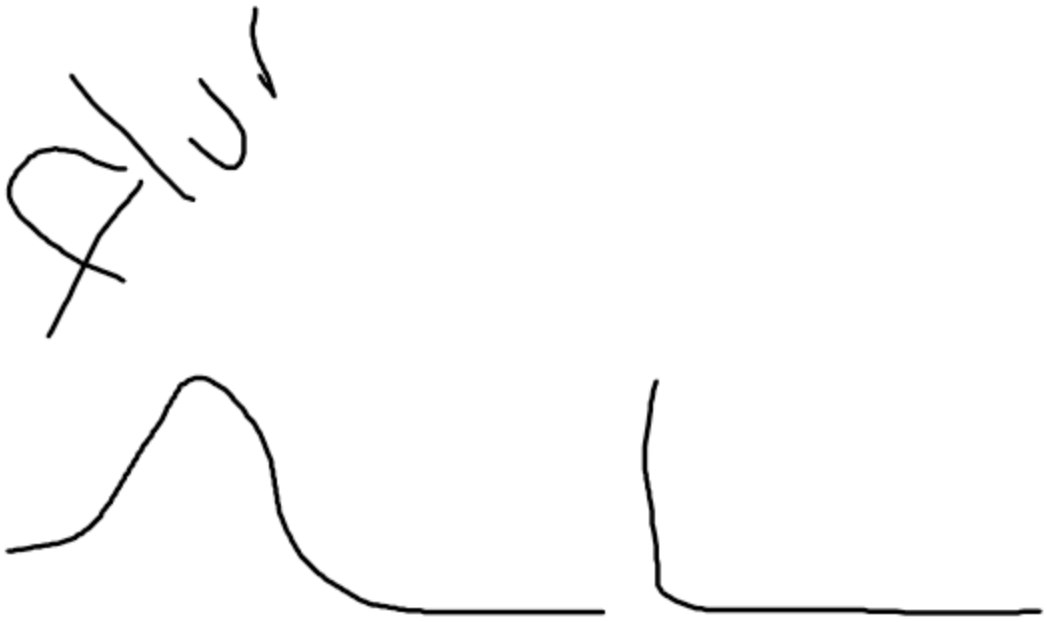
-add restriction sites to cut base pairs--> creates overhang

-want to PCR to fragments that you want

Usual PCR

Golden Gate PCR

Group probably wants to work more with golden gate technique

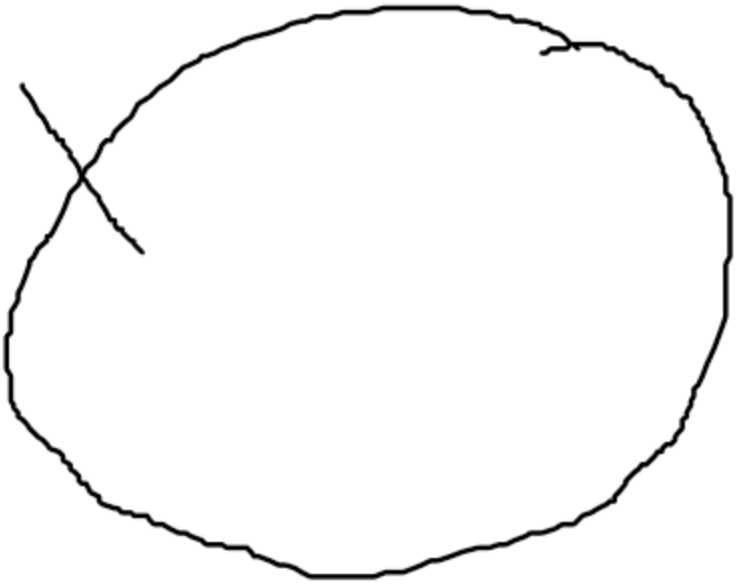


↳

captains

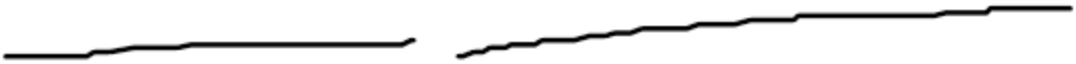
Digests present

"*.



No ends from digest left behind

me



June 16, 2016

Thursday, June 16, 2016
3:50 PM

Today, we spent most of the time working with promoter and primer design (with Steve)

Promoter is the part before the gene that initiates transcription. We are trying to find the promoter sequence so that we can insert it as a primer in order to do PCR and amplify the part of the DNA that we want.

Detailed procedure for finding promoter sequence:
-use the identifier number to find the gene of interest in NCBI database. Once you select the gene, you will find multiple genes that originate from the same sequence.
-we want the promoter range (ie range between one gene and another) to be over 100 base pairs

-find the location of where one gene ends and find the location of where the next gene begins. Use these numbers to place into GenBank (ex: http://www.ncbi.nlm.nih.gov/nuccore/NC_000913.3?report=genbank&from=1279864&to=1283607)

If can't find exact bp number of location of genes using ncbi, then can use ecocyc database and go to operon tab to find the gene location by hovering over the structures

-copy and paste code into ApE to get sequence. You want the sequence presented to show parts of the end of one gene and the beginning of the next one, so adjust the numbers in GenBank and transfer code a few times into ApE until you get the result you want

-once one gene is highlighted at one end and another gene is highlighted at another, that verifies that the sequence in the middle is entirely the promoter sequence

-can check that the promoter exists in igem registry by pasting in gene sequence and comparing with all parts

Detailed procedure to find forward primer (ie need to have found promoter sequence first):

-select about the first 20 base pairs from the promoter sequence and insert in NEB Tm calculator (http://tmcalculator.neb.com/#!/) under primer 1. make sure everything looks good and it doesn't give an indication to whether it's too long or short

This actually indicates the temp needed to work with the primer in order for it to anneal

-adjust as needed and whatever is works is the forward primer

…reverse primer:

-use about last 20 base pairs of promoter sequence and right-click them and select "copy reverse primer" (on ApE)

-paste result into primer 2 of NEB Tm calculator

-if given errors, etc adjust accordingly (delete base pairs, etc), otherwise if everything works, then that is your reverse primer

Make sure that if you have to delete bp from NEB Tm primer 2 from the end of the sequence, then those bp correspond to the first bp you selected from the promoter sequence in ApE and they need to be deleted

Links:
http://parts.igem.org/Part:BBa_M33001
http://www.ncbi.nlm.nih.gov/gene/?term=B1224[gene%20name]
http://ecocyc.org/gene?orgid=ECOLI&id=EG10638#tab=TU

June 21, 2016

Tuesday, June 21, 2016
1:19 PM

We worked on gel extraction kit. We ran the gel for with 14A, 14C, and 1P (promoter). We were able to isolate 1P and perform gel extraction on it. 14A and 14C were too diffused to perform gel extraction.

June 22, 2016

Wednesday, June 22, 2016
2:09 PM

Weekly group meeting

Discussed gel extraction problem with diffused …… solution is to use PCR method

Time sensitive promoter
Active at different points, for exampleif you have a pathway with 5 genes, you want the first gene to be expressed first before gene 4 and 5 are expressed, so ideally you would use an early growth phase promoter for the first gene and a late growth phase promoter for genes 4 and 5

Validating promoter with GFP

To do:
Read through original paper
Build on one of application
Looking through previous wikis
Definitions: biobrick

June 28, 2016

Tuesday, June 28, 2016
11:50 AM

PCR procedure for promoters:

1. Place primers (forward and reverse) and master mix on ice
2. Label other smaller tubes and place the following:
    a. 1uL of genomic DNA
        i. We measured concentration of DNA with nanodrop and got 119.2ng/uL. Adding 1uL of DNA was good enough for this mix
    b. Add water so total in each tube is 25uL (9uL of water)
    c. 1.25uL of each forward and reverse primer AFTER dilution
        i. Do NOT add this much of primer! It needs to be diluted first in a 1:10 ratio. So in other tubes add 2uL of primer and 18uL of water. From this take 1.25uL and add to mix
    d. Add 12.5uL of master mix as the last thing
3. Place in thermocycler and make sure temp setting is correct. Temp at gradient setting should be 5 degrees lower than melting temp
4. While waiting for thermocycler, want to set up gel for confirmation of DNA to make sure the length is as expected

Gel electrophoresis (for one gel):

1. Add .5g agarose and 50uL of TAE buffer in bottle
2. Place in microwave for 1 minute, but take out every 15 seconds to shake liquid around so agarose can better dissolve
3. Once liquid is clear, let cool for 5 min
4. Add 6uL of advanced midori green dye
5. Place liquid in gel box and make sure lanes chip is set. Use lane chip with the most wells so that the smallest volume can be used from the primer. Liquid level shouldn't exceed the top of the lanes
6. Wait for about 30 min for gel to harden
7. Place gel in electrophoresis kit
8. Loading dye
    a. Our loading dye was 6X so added 1uL of dye and 5uL of DNA that came from thermocycler from each tube into 3 other tubes
    b. Don't want the entire DNA to be imaged, so only get portion of DNA on gel
9. With gel submerged in TAE buffer, add ladder to first lane, then each of tubes with loading dye and DNA, then last lane is ladder again. Since using lane chip with about 15 lanes, want to add about 5uL to each lane
10. Set to 120V for 20min
11. Once completed, image

June 30, 2016

Thursday, June 30, 2016
12:35 PM

Performed PCR on promoter 3321 with Caroline using same steps from June 28

Nanodrop data for promoter 3321 (ng/uL):
Tube 1: 723.5
Tube 2: 515
Tube 3: 410.4

July 1, 2016

Friday, July 1, 2016
12:42 PM

Today, Caroline and I performed PCR on promoters 220 and 871. we cast them on the gel to make sure the length coincides with what is expected

July 12, 2016

Tuesday, July 12, 2016
2:27 PM

Today, Viraat and I performed PCR on GFP construct (similar to procedure on June 28, but still some differences).

1. Used iGEM20 and 21 as forward and reverse primers. We diluted each one in different tubes (1:10 ratio) then took 1.25uL of each and placed in master tube.
2. We have been using about 100ng of DNA in the total tube, so the gblock GFP construct/gene fragments that we used was set at 10ng/uL, meaning that we added about 10uL of the GFP construct.
    a. We did NOT add any genomic DNA because the construct includes the DNA itself
3. Add 12.5uL of master mix to master tube with all mixture
    a. Didn't need to add any water because the total tube volume ended up being 25uL
4. Place in thermocycler setting "gfp00short" and set a temperature gradient of 57-63C
5. Prepare gel to run pcr product and get result
    a. Should expect fragments to be about 1100 base pairs (I think…)

NOTE: we originally made 2 gels to include PCR primer products from Viraat and Hiba's group yesterday, but one of the gels was flimsy near the wells so we didn't use it. The following was inserted in lanes on gel from left to right:

Gblock GFP construct I, II, III	IGEM 8/9 I, II, III	DNA ladder	IGEM 12/13 I, II, III	IGEM 22/23 I, II, III

Result: they were pretty good! Each 3 lanes were all at the same level, and IGEM 8/9 and 22/23 had very prominent bands. IGEM 12/13 didn't have as much DNA so its bands weren't as clear as the rest. The gblock DNA was positioned correctly with respect to other bands because the construct had bands near 1000bp and the other primers had bands around 400bp, which is further proves that this was successful!

Tomorrow we plan to do traditional cloning using protocol on this page:
http://parts.igem.org/Help:Protocols/Linearized_Plasmid_Backbones

The only problem with this protocol is that it corresponds to two restriction sites, but the construct we are using only has one restriction enzyme site, so we have to know which enzyme to use. To do this, we have to look through the sequence of the construct, and then use NEB or the likes to insert the prefix, promoter of GFP part, GFP gene, then suffix to be from 5'-3' to figure out which restriction enzyme to use.

July 13, 2016

Wednesday, July 13, 2016
9:49 AM

Need to insert PCR amplified gblock into vector before cloning.

Obtain sequence of gblock (prefix, suffix, gfp promoter, gfp gene, terminator) to find single restriction enzyme to use
Once restriction enzyme found, follow directions in

For transformation and making LB plates, do we pour LB broth into plates and have to sit overnight? Do we already have plates made and where?

Today after the group meeting, Steve checked our sequence to make sure the enzymes listed in the protocol were accurate.
To do:
-brainstorm ideas for sweetcorn festival
-if kit doesn't have plate, then follow up with Todd or Michelle to discuss what type of plate is needed for plate reader

July 14, 2016

Thursday, July 14, 2016
12:37 PM

- PCR purification kit

-to remove any mutations that may have occurred, etc we need to find

http://parts.igem.org/Help:Protocols/Linearized_Plasmid_Backbones
Traditional cloning (placing gblock into plasmid backbone)

- Digest:

1. Create enzyme master mix (25uL total)-- Steve checked our sequence to make sure that the enzymes we are using correspond to the correct restriction sites we are trying to reach
    a. 5uL Cutsmart
    b. .5uL BSA
    c. .5uL EcoRI-HF
    d. .5uL PstI-HF
    e. 18.5uL of H2O
2. Add 4uL of linearized plasmid backbone (25ng/uL for 100ng total)
3. Add 4uL of enzyme master mix
4. Digest in incubator at 37C for 30 min
5. Heat kill at 80C for 20 min

July 15, 2016

Thursday, July 14, 2016
4:59 PM

- Digestion(in preparation for Ligation):
- Add equimolar EcoRI PstI digested fragment
  a. http://parts.igem.org/Help:Protocols/3A_Assembly
  b. Equimolar to amount of DNA, so concentration using nanodrop was 23.6ng/uL
  c. Digested fragment (ie enzyme master mix) includes:
    - 5 ul Cutsmart
    - 0.5 ul BSA
    - 0.5 ul EcoRI-HF
    - 0.5 ul PstI-HF
    - 18.5 ul dH20

From <http://parts.igem.org/Help:Protocols/3A_Assembly>

These enzymes were checked with the gblock sequence to make sure that one insert was going to take place, instead of using 4 enzymes to make 4 cuts and give us two inserts (we only wanted one insert!)

- Then to digest add 4uL of this to 4uL of PCR purified gblock (combined all 3 tubes since had same properties given result from gel imaging)
- Digest at 37C for 30min-------- RUN AT LEAST 2 HOURS!!!! LONGER RUN TIME, MORE CUTS
  a. Used water bath
- Heat kill at 80C for 20 min

This procedure took long until we understood what the first step was asking from us. Also when we finished this second master mix, instead of using the PCR purified gblock, the pre-PCR purified gblock was digested and heat killed. We kept the result, but had to do the procedure again using PCR purified gblock.

Our PCRed, purified, and now digest gBlock is labeled "gBlock Digest" and has been placed in the Ligation Box.

July 18, 2016

Monday, July 18, 2016
2:45 PM

Had to repeat digested plasmid backbone step because was accidently thrown out before the weekend…need to label better…when digested again, used water bath

1. Add 2uL of digested plasmid backbone (25ng total) - "Plasmid Digest 7/18"
2. Add equimolar EcoRI PstI digested fragment - "gBlock Digests I, II, and III"
   1. Concentration was 23.6ng/uL using nanodrop, so will use 2uL of digested fragment
3. Add 1uL of T4DNA ligase buffer (not quick ligase)
4. Add .5uL of T4 DNA ligase

5. Add water to make total 10uL
6. Ligate in 16C for 30 min--AT LEAST 2 HOURS, MAYBE OVERNIGHT
    1. Used thermocycler
7. Heat kill at 80C for 20 min

Product labeled under "Ligation 7/18 I, II, and III"

Not sure about steps for transformation, so ask and work on it tomorrow

DO CONTROL WHERE INSTEAD OF GBLOCK DIGEST ADD WATER (IWTHOUT INSERT)
MIX DNA AND DO ANNEALING PROTOCOL (THERMOCYCLER SET AT 80 AND COOL TO 4C AT .1C/SEC, THEN ADD BUFFER AND LIGASE
THERMOCYCLER: CYCLE BETWEEN 37 AND 16??

July 19, 2016

Tuesday, July 19, 2016
2:48 PM

- Transformation (WRONG, SHOULDN'T USE E COLI COLONIES):
  Taken from: https://www.addgene.org/plasmid-protocols/bacterial-transformation/
- Take out plates with E. Coli colonies
- In 1.5 mL microcentrifuge tube, add 50uL of LB media
- Then streak a lot of cells from the plate with E Coli colonies (from the 4C freezer) with a tip and swirl it in LB media
- Add 1uL of DNA that was ligated yesterday---ADD UP TO 5UL FOR BETTER EFFICIENCY
- Place on ice for 30 min
- Heat shock in 42C water bath for 45 sec
- Put tubes back on ice for 2 min
- Add 500uL of LB media
- Place in shaking incubator at 37C for 45 min--UP TO AN HOUR
- Place 50uL or 75uL of transformation on LB agar plates --PLATE UP TO 200UL
    1. These were found in 4C freezer And were labeled "iGEM LB+CM 6-1-2-16"
    2. Made 2 plates for each of ligated plasmids: added either 50 or 75 uL
- Place some beads on each plate and shake to distribute cells evenly
- Incubate in 37C overnight

Transformation tubes are stored in 4C freezer

NOTES IN CAPS ARE FEEDBACK FROM TODD

July 20, 2016

Wednesday, July 20, 2016
1:38 PM

Grow cells over 24 hours
-get fluorescence over OD measurement
-have 120-200uL of liquid per well
-have control using cells without gfp to check autofluorescence from cell or media
    use buffer to wash
-measurements:
    Fluorescence: excitation and emission wavelength
    OD: wavelength

If don't want to use plate reader, then can use tube (for 5mL) or flask (greater than 25mL)
-would take tests at 0 hours, 2, 8, and 24 hours

Add negative control: add backbone, ligase, but NO insert (ie only digested backbone)

Colony pcr: Take colony and place in 100uL of LB , take 1uL as template for PCR
Use primers for backbone

July 21, 2016

Thursday, July 21, 2016
2:17 PM

With help from Todd, we were able to correct our transformation protocol from the one we used on July 19

- Transformation (CORRECTED):

Taken from: https://www.addgene.org/plasmid-protocols/bacterial-transformation/

- Take out DH5-alpha competent cells from -80C freezer and thaw on ice for about 20 min
- Place agar plates with antibiotic in 37C incubator
- In 1.5 mL microcentrifuge tube, add 50uL of competent cells.
- Add 5uL of DNA that was ligated (labeled "ligation", there are 3 of them) to microcentrifuge tube
- Place microcentrifuge tube on ice for 20-30min
- Heat shock in 42C water bath for 45 sec
- Place tubes on ice for 2 min
- Add 500uL of LB media
- Place in shaking incubator at 37C for 1 hour
- Plate 200uL of transformation on LB agar plates
    1. These were found in 4C freezer And were labeled "iGEM LB+CM 6-1-2-16"
- Place about 4 beads on each plate and shake to distribute cells evenly
- Incubate in 37C overnight

Transformation tubes are stored in 4C freezer under "transformation 7-21"

July 25, 2016

Monday, July 25, 2016
1:33 PM

2uL of competent cells on LB plate (no antibiotic) to prepare to make more competent cells tomorrow
Placed in 37C incubator

The point of this transformation is to see if the cells we used were actually competent since our original transformation didn't develop any colonies.

- Transformation (CORRECTED with Michelle):

Taken from: https://www.addgene.org/plasmid-protocols/bacterial-transformation/

- Take out DH5-alpha competent cells from -80C freezer and thaw on ice for about 20 min
- Place agar plates with antibiotic in 37C incubator
- In 1.5 mL microcentrifuge tube, add 50uL of competent cells.
- Add 5uL of pUC19 Control DNA from Michelle to microcentrifuge tube
- Place microcentrifuge tube on ice for 20-30min
- Heat shock in 42C water bath for 45 sec
- Add 500uL of LB media
- Place in shaking incubator at 37C for 1 hour
- Plate 200uL of transformation on LB agar plates
    1. These were found in 4C freezer And were labeled "iGEM LB+CM 6-1-2-16"
- Place about 4 beads on each plate and shake to distribute cells evenly
- Incubate in 37C overnight

Transformation tubes are stored in 4C freezer under "transformation 7-25"

Sterile CaCl and put in 4C freezer, should be cold
All to be done in ice
Everything done night before.

July 26, 2016

Tuesday, July 26, 2016
1:22 PM

CAROLINE IS BACK!

Lycopene parts arrived. It has an agar stab, so today we will mix the part and streak it on a plate that has ampicillin

Digest

pSB1C3 backbone digest:

1. 2uL Cutsmart
2. 1uL EcoRI-HF
3. 1uL PstI-HF
4. 16uL linearized plasmid backbone pSB1C3

- Total reaction: 20uL, set at 20ng/uL

GFP digest (insert):

1. 4uL Cutsmart
2. 2uL EcoRI-HF
3. 2uL PstI-HF
4. 32uL gblock digest
    a. At this point, we combined all 3 tubes into one tube that had a little less than 100uL left
    b. Nanodrop concentration: 15.5ng/uL

- Total reaction: 40uL at about 20ng/uL

Do PCR purification kit on backbone and GFP digest

Making LB plates:

1. 10g Bacto Tryptone
2. 10g NaCl
3. 5g Yeast extract
4. 7.5g agar THIS IS ACTUALLY WRONG! SHOULD HAVE BEEN 15G OF AGAR!!
5. 1000mL of DI water
6. Autoclave
    a. Separate into two tubes so mixture doesn't overflow (so two-500mL mixtures)
7. Let it cool to room temperature
8. Split tubes and place according to table below
    a. 500mL of LB + 625uL of chloromphenicol
    b. 250mL of LB + 250uL of ampicillin
    c. 250mL of plain LB

Antibiotic	Desired LB Plate Concentration	Antibiotic Concentration	Volume of LB Broth	Mass of Antibiotic Necessary	Volume of Antibiotic Necessary
Ampicillin	100 ug/mL = 0.100	100 mg/mL	250 mL	25 mg	250 uL

	mg/mL				
Chloramphenicol	25 ug/mL = 0.025 mg/mL	20 mg/mL	500 mL	12.5 mg	625 uL

9. Plate
10. Leave to solidify for 30-45 minutes
11. Flip plates over and keep overnight

July 27, 2016

Wednesday, July 27, 2016
1:36 PM

Plate lycopene parts:

- We plated lycopene parts (BBa_K118013 and Bba_K118014) and placed in 37C incubator

Notes:
Yesterday in the PCR purification, we were supposed to elute with 20uL instead of 50uL. So Caroline realized that our DNA was very dilute so she took a few steps to remove some liquid

Making competent cells:

1. Place 50uL of old competent cells in 5mL of LB in tube and keep in incubator overnight
2. Prepare .1M of CaCl2
   a. .1M CaCl2 --> .1mol/L * .03 L * 111g/mol = **.333g of CaCl2**
   b. Will need that amount of CaCl2 to make .1M

1. Make 1% inoculation to 50mL LB
   a. Ie add 500uL of culture that was kept overnight
   b. Place in shaker at 37C until OD is at .5 (about 1.5 hours)
   c. Transfer 50mL culture to 50mL falcon tube
   d. Centrifuge at 3000rpm for 10 min at 4C
   e. Remove as much supernatant as possible
   f. Resuspend cells in 5mL

Ligation:

1. 7uL gblock digest
2. 1.5uL backbone digest
3. .5uL ligase
4. 1uL buffer

- Reaction mix: 30ng

5. Place in thermocycler at 10C for 30sec then 30C for 30 sec and cycle for about 16 hours.

July 28, 2016

Thursday, July 28, 2016
12:34 PM

Transformation of GFP and backbone into NEB C29871 high efficiency competent cells:

1. For C2987I: Thaw a tube of NEB 5-alpha Competent *E. coli* cells on ice until the last ice crystals disappear. Mix gently and carefully pipette 50 µl of cells into a transformation tube on ice.
    a. Caroline suggested we set up 4 tubes:
        i. 1uL control pUC19 control plasmid which will grow on plain LB (control)
        ii. 1uL GFP plasmid which will be grown on LB + chloramphenicol plate
        iii. 2uL GFP plasmid which will be grown on LB + chloramphenicol plate
        iv. Plain LB + ampicillin plate with no plasmid (control so see if pUC19 will grow since it has ampicillin resistance)
2. Add 1–5 µl containing 1 pg–100 ng of plasmid DNA to the cell mixture. Carefully ick the tube 4–5 times to mix cells and DNA. Do not vortex.
3. Place the mixture on ice for 30 minutes. Do not mix.
4. Heat shock at exactly 42°C for exactly 30 seconds. Do not mix.
5. Place on ice for 5 minutes. Do not mix.
6. Pipette 950 µl of room temperature SOC into the mixture.
7. Place at 37°C for 60 minutes. Shake vigorously (250 rpm) or rotate.
8. Warm selection plates to 37°C.
9. Mix the cells thoroughly by icking the tube and inverting, then perform several 10-fold serial dilutions in SOC.
10. Spread 50–100 µl of each dilution onto a selection plate and incubate overnight at 37°C. Alternatively, incubate at 30°C for 24–36 hours or 25°C for 48 hours.

From Augustines notebook:

As suggested by Caroline, I set up 4 tubes. (1)Contains 1uL control pUC19 control plasmid (2) contains 1uL GFP plasmid (3) contains 2uL GFP plasmid (4)Contains no plasmid. (1) and (4) are controls. We grow (1) on plain LB, (2) and (3) on Chloramphenicol LB and (4) on Ampicillin LB (as the pUC19 plasmid has ampicillin resistance).

Lycopene parts:

1. In tubes of 5mL LB, add 5uL of ampicillin

2. From culture plates incubated overnight, take a colony with tip and drop tip in tube
3. Place tubes in shaking incubator overnight

August 1, 2016

Monday, August 1, 2016
2:21 PM

- Transformation (CORRECTED with Michelle):

Taken from: https://www.addgene.org/plasmid-protocols/bacterial-transformation/

- Take out new DH5-alpha competent cells from -80C freezer and thaw on ice for about 20 min
- Place agar plates with antibiotic in 37C incubator
- In 1.5 mL microcentrifuge tube, add 50uL of competent cells.
- Add 5uL of pUC19 Control DNA from Michelle to microcentrifuge tube
- Place microcentrifuge tube on ice for 20-30min
- Heat shock in 42C water bath for 45 sec
- Add 500uL of LB media
- Place in shaking incubator at 37C for 1 hour
- Plate 200uL of transformation on LB agar plates
    1. These were found in 4C freezer And were labeled "iGEM LB+amp new DH5a cell transformation 8-1-16"
- Use two tips to streak cells on plate in both directions
- Incubate in 37C overnight

Transformation tubes are stored in 4C freezer under "new DH5a cells transformation 8-1"

Result: successful!

August 2, 2016

Tuesday, August 2, 2016
2:55 PM

- DH5a Competent cell Transformation:-

The transformation from yesterday worked well. Colonies grew on the plate, so that means the new DH5a competent cells work.

- Testing GFP:

From the gblock PCR purified construct that Caroline prepared, I ran a gel to make sure the length was at about 1000 base pairs. In the leftmost lane, I added 7uL of ladder. Two lanes over, I added 6uL of the mixture that included 1uL of loading dye (6X) and 5uL of PCR purified gblock.

The results weren't very promising. The ladder came out strong, but the construct was a fuzzy black region at around .8 - .9 kb (instead of the expected 1 kb). Todd mentioned that the cycle number we used was too low (10), so we will run another PCR with the gblock at 25 cycles

PCR protocol:

(from July 12, 2016)

- Used iGEM20 and 21 as forward and reverse primers. We diluted each one in different tubes (1:10 ratio) then took 1.25uL of each and placed in master tube.
  a. In separate tubes, added 2uL of each primer and 18uL of water
- Add 1uL of diluted gblock (set at 1ng/uL, to get total of 1ng of gblock, Caroline had some of this already prepared)
- Add 10.25uL of water (to make total mixture add up to 25uL)
  a. Didn't add this the first time around, so had to do it again
- Add 12.5uL of master mix to master tube with all mixture
  a. FIRST TRY: Forgot to add water to make the reaction mix to 25uL. For this mixture, the total volume per tube was 14.75uL
- Place in thermocycler setting "gfp00short" but change from 10 cycles to 25 cycles
- Prepare gel to run pcr product and get result
  a. Should expect fragments to be about 1000 base pairs (1kb for 2 log ladder)

While waiting for PCR, I prepared a gel:

Gel electrophoresis (for one gel):

(from June 28, 2016)

- Add .5g agarose and 50uL of TAE buffer in bottle
- Place in microwave for 1 minute, but take out every 15 seconds to shake liquid around so agarose can better dissolve
- Once liquid is clear, let cool for 5 min
- Add 6uL of advanced midori green dye
- Place liquid in gel box and make sure lanes chip is set. Use lane chip with the most wells so that the smallest volume can be used from the primer. Liquid level shouldn't exceed the top of the lanes
- Wait for about 30 min for gel to harden
- Place gel in electrophoresis kit
- Loading dye
  a. Our loading dye was 6X so added 1uL of dye and 5uL of DNA that came from thermocycler from each tube into 3 other tubes
  b. Don't want the entire DNA to be imaged, so only get portion of DNA on gel
- With gel submerged in TAE buffer, add ladder to first lane, then each of tubes with loading dye and DNA, then last lane is ladder again. Since using lane chip with about 15 lanes, want to add about 5uL to each lane
- Set to 120V for 20min
- Once completed, image

The first time I ran the PCR I forgot to add water to make mixture add up to 25uL ("gblock PCR 8-2 MS"). So redid it and the correct tubes are labeled "gblock PCR #2 8-2 MS"

On the gel ("2016-08-02 120V 20min gblock PCR #2 Mariam") starting from the rightmost lane is ladder, then 3

Result: not very successful. The bands aren't very bright and shorter than we expected

August 3, 2016

Wednesday, August 3, 2016
1:54 PM

Since we are having problems with our PCR, Todd recommended we use Buffer G instead of the Q5 polymerase. Buffer G is a more robust method to use.

2X Buffer G PCR:

1. Add 25uL buffer G
2. .25uL Q5 polymerase
3. 2.5uL forward primer (10uM)
4. 2.5uL reverse primer (10uM)
5. 20uL water
6. .5uL template (ie gblock GFP construct
7. Place in thermocycler with "gfp00short" setting set at 30 cycles and temperature ranging from 63.5-65.5C

The first time we did it, we set the thermocycler with temperature range from 58-61 ("GFP buffer G PCR 8-3" tube 1 at 58C and tube 2 at 61). There weren't very clear streaks, so we did it again with a higher annealing temperature.

The second time we did it the temperature range was from 63.5-65.5C the tubes are stored in the -20C freezer in the PCR products box and titled "GFP buffer G #2 8-3" with blue markings at the top. The gel results weren't significantly better than the last time. Similar streaks and there weren't any visible bands where we wanted them to be at 1kb.

August 4, 2016

Thursday, August 4, 2016
1:21 PM

We are running the Q5 PCR procedure using Touchdown PCR, which uses cycles through an annealing temperature higher than the optimal melting point temperature. It starts 10 degrees higher than the optimal melting point temperature and decreases by one degree after each cycle.

(Touchdown) PCR protocol:

(from July 12, 2016)

1. Used iGEM20 and 21 as forward and reverse primers. We diluted each one in different tubes (1:10 ratio) then took 1.25uL of each and placed in master tube.
   a. In separate tubes, added 2uL of each primer and 18uL of water to make 1:10 dilutions
2. Add 1uL of diluted gblock (set at 1ng/uL, to get total of 1ng of gblock, Caroline had some of this already prepared)
3. Add 10.25uL of water (to make total mixture add up to 25uL)
4. Add 12.5uL of master mix to master tube with all mixture
5. Place in thermocycler setting "TOUCHDOWN" for the touchdown PCR settings.
   a. The average optimal melting temperature was 64 so we had the initial annealing temperature set to 75C
   b. http://bitesizebio.com/2203/touchdown-pcr-a-primer-and-some-tips/
6. Prepare gel to run pcr product and get result
   a. Should expect fragments to be about 1000 base pairs (1kb for 2 log ladder)

Results: SUCCESSFUL for most part…!

Tubes 1 and 2: There were bands in the 1kb region, though the tube labeled 1 had clearer bands.
Tubes 3 and 4: Only tube 3 exhibited a band at the 1kb region. For some reason tube 4 didn't have anything even though its concentration was much higher

performed PCR purification kit on all tubes

Nanodrop results:

Tube 1:	9.6 ng/uL
Tube 2:	5.6 ng/uL
Tube 3:	2.6 ng/uL
Tube 4:	7.3 ng/uL

All tubes are stored in the PCR products box and labeled "PCR purified gblock TD-PCR 8-4" and have their corresponding concentration and number
(TD-PCR stands for TouchDown-PCR)

August 8, 2016

Monday, August 8, 2016
1:41 PM

Today we were supposed to start Gibson assembly but the backbone PCR reaction didn't work out the first time because the primers were stuck to each other. We tried it again with touchdown PCR and the result was 0 ng/uL and there was nothing on the gel :(

August 9, 2016

Monday, August 8, 2016
4:20 PM

Today I am working on a Gibson assembly between the GFP and backbone. The Gibson assembly protocol is the same as the one found here on page 12 of this document: https://www.neb.com/~/media/Catalog/All-Products/709D232D72C045D2B2B1089A89DC879F/Datacards%20or%20Manuals/manualE2621.pdf. This Gibson assembly will be used to compare with the ligation to see which one produced more/better product

Caroline PCRed the backbone, so we had to run it on the gel and PCR purify it before using it

Gibson assembly protocol:

1. Make sure all parameters are met in the table
   a.

Backbone (vector): 2000bp	40ng
GFP (insert): 1000bp	40ng

   b. We have two fragments so the DNA ratio is supposed to be 1:2, which turns out to be the same in terms of amount for each
   c. Total amount of fragment: 1000bp of DNA==1.52pmol/ug
      i. 40ng of DNA--> .0608pmol x 2 = .1216pmol so suffices
2. Add 4uL of *original* GFP gene fragment insert, not PCRed or anything, straight from IDT (set at 10ng/uL and want 40ng)
3. Add 4uL of backbone (after gone through PCR and PCR purification)
   a. Concentration: 10.6 ng/uL
4. Add 10uL of DNA assembly master mix
5. Add 2uL of water to reach total volume of 20uL
6. Place in thermocycler at 50C for 15 min
7. Store on ice in -20C

Gibson assembled part is placed in PCR products box labeled "Gibson assembly 8-9"

- Transformation:

Taken from: https://www.addgene.org/plasmid-protocols/bacterial-transformation/

1. Take out new DH5-alpha competent cells from -80C freezer and thaw on ice for about 20 min
2. Place agar plates with CM antibiotic in 37C incubator
3. In 1.5 mL microcentrifuge tube, add 50uL of competent cells.
4. Add 2uL of gibson assembled part to microcentrifuge tube

5. Place microcentrifuge tube on ice for 20-30min
6. Heat shock in 42C water bath for 45 sec
7. Add 500uL of LB media
8. Place in shaking incubator at 37C for 1 hour
9. Use two tips to streak cells on plate in both directions
10. Incubate in 37C overnight

Gibson assembled transformed part is labeled "Gibson assembly transformation 8-9" and can be found in the PCR products box next to the other Gibson assembled part

Result:

Sweetcorn festival contact: 2173443872

August 15, 2016

Monday, August 15, 2016
3:44 PM

Today, augustine and I inoculated the Gibson constructs (0-fold and 1-fold) as well as the 5 interlab study parts. There were few number of colonies in the positive control of the interlab study part and almost no colonies in the 1-fold of the Gibson construct

We added 5mL of LB and 8.75uL of CM in a glass tube and then placed a tip of inoculated colony. The tubes are in the shaking incubator overnight.

Updates:
-We need to make more CM and plates, but we couldn't find CM powder anywhere, so we will need to order them from online.
-sweetcorn festival: we need to bring our own table, chairs, tent, etc
-less than a week until Quad day

August 17, 2016

Wednesday, August 17, 2016
1:27 PM

1. Inoculate interlab colonies: 2 tubes for each device
    a. Placed in shaking incubator at around 3pm
2. Transform gfp and puc19 to measure fluorescence of our constructs
    a. Only transformed Gibson GFP and ligation colony 1 because there were only two plates left with CM

3. Send gfp for sequencing
    a. Primer concentration: 10uM for 1:10 dilution
    b. Gibson: 240ng/uL
    c. Ligation colony 1: 63ng/uL
    d. Ligation colony 2: 67ng/uL
    e. Ligation have low yields so need to do some miniprep tomorrow to increase concentration

August 18, 2016

Thursday, August 18, 2016
12:54 PM

Today, we are taking growth measurements of the interlab study. Measurements need to be taken once every hour for 6 hours.

I also made LB +CM plates since we ran out

Tomorrow, we will send everything for sequencing

September 16, 2016

Friday, September 16, 2016
5:44 PM

Today, Augustine and I worked on PCR purifying promoters 4202, 3321, 1304 and the linearized backbone that Jonathan was working on last.

Used Qiagen PCR purification kit:
Only step we didn't follow was the elution. We eluted with 30uL of water instead of buffer

Digestion:
Followed linearized backbone protocol from iGEM website

For enzyme master mix:-
-used Cutsmart instead of NEB Buffer 2
-Used EcoRI-HF and PstI-HF
-we did add DpnI

Linearized backbone concentration from nanodrop (was split into 2 tubes):

122.2 ng/uL	161 ng/uL

Digestion:
Took 1uL of linearized backbone and 4uL of enzyme master mix --> digested in 37C bath for 30 min

Since we used -HF enzymes, they are not affected during the heat kill step, so we need to use the PCR purification kit on the digest instead of heat killing

September 21, 2016

Wednesday, September 21, 2016
5:32 PM

I worked on redigesting promoter 30, 32, and 34.

Promoters:

Enzyme Master Mix for promoter 30, 32, 34 (25ul total, for 5 rxns)

- 5 ul Cutsmart
- 0.5 ul BSA
- 0.5 ul EcoRI-HF
- 0.5 ul PstI-HF
- 18.5 ul dH20

- Add 8 ul of promoter to 3 separate tubes (25ng/ul for 200ng total)
- Add 8 ul of Enzyme Master Mix to each tube
- Digest 37C/30 min
- Perform PCR purification since used HF enzymes

From <http://parts.igem.org/Help:Protocols/Linearized_Plasmid_Backbones#Digest>

Backbone:

- Enzyme Master Mix for Plasmid Backbone (25ul total, for 5 rxns)
- 5 ul Cutsmart
- 0.5 ul BSA
- 0.5 ul EcoRI-HF
- 0.5 ul PstI-HF
- 0.5 ul DpnI (Used to digest any template DNA from production)
- 18 ul dH20

- Add 8 ul linearized plasmid backbone to 2 separate tubes (25ng/ul for 200ng total)
- Add 8 ul of Enzyme Master Mix to each tube
- Digest 37C/30 min
- Perform PCR purification since used HF enzymes

From <http://parts.igem.org/Help:Protocols/Linearized_Plasmid_Backbones#Digest>

Nanodrop concentrations: LOW YIELDS :(

Batch 1 linearized backbone: 2.6 ng/uL
Batch 2 linearized backbone: 2.8 ng/uL
Promoter 30: 3.8 ng/uL
Promoter 32: 4 ng/uL
Promoter 34: 6.5 ng/uL

September 28, 2016

Wednesday, September 28, 2016
11:33 AM

Did the following this morning:

1. Ran PCR on backbone
    a. Used Q5 protocol which consisted of:
        i. 2.5ul of SB-prep-3P-1
        ii. 2.5 ul of SB-prep-2Ea
        iii. 2 ul of template dna (diluted 14A at 1.47 ng/uL for total of about 3 ng of dna)
        iv. 18 ul nuclease free water (borrowed from guy from BSD)
        v. 25 ul Q5 polymerase
    b. Placed in thermocycler with same settings as in PCR program under single reaction - http://parts.igem.org/Help:Protocols/Linearized_Plasmid_Backbones#Digest
2. Digested 14A
    a. .5 Psti-HF
    b. .5 EcoRI-HF
    c. 5 ul cutsmart
    d. 19 ul water
    e. Took 5 ul of that mix and added 5 ul of 14A at 147 ng/uL to get about .7 ug of dna
    f. Digested in 37C for 30 min
    g. Want to verify on gel with uncut sample and ladder
        i. RESULTS: gave 2 bands but the longer one was thicker than the other
3. Digested promoter 36, 38, 40, 42, 44, 46
    a. For 36, 42, 44, 46: used 10 uL of master mix and 10 ul of dna
    b. For 38: used 15 ul of each
    c. For 40 and 6G (64.4ng/ul): used 20 ul of each
    d. Placed in water bath then transfer to freezer

September 30, 3016

Friday, September 30, 2016
7:10 PM

Today, augustine and I worked on digesting the backbone and doing a gel extraction for the promoters.

Gel extraction:

-we ran all 6 promoters (36, 38, 40, 42, 44, 46) on a gel and used the gel extraction kit. Once that was done (Augustine was finishing it up), it will be ready to be digested.
-results: image is saved on the computer. The control with 14A had 2 bands. The promoters had bands around 200 base pairs

Digesting backbone:
-we followed the digestion protocol from iGEM by using the following

a. .5 Psti-HF
b. .5 EcoRI-HF
c. .5 DpnI
d. 5 ul cutsmart
e. 19 ul water
f. Took 4 ul of that mix and added 4 ul of backbone at about 480 ng/uL to get about 2 ug of backbone
g. Digested in 37C for 30 min
h. Pcr purify (not sure if Augustine did this step, I had to leave early)

Promoters and backbone are ready for ligation (if pcr purification was done on the backbone)